Ca\(^{2+}\)/Calmodulin-Dependent Protein Kinase Regulates GABA-Activated Cl\(^{-}\) Current in Cockroach Dorsal Unpaired Median Neurons

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Alix, Philippe, Françoise Grolleau, and Bernard Hue. Ca\(^{2+}\)/Calmodulin-dependent protein kinase regulates GABA-activated chloride current in cockroach dorsal unpaired median (DUM) neurons. J Neurophysiol 87: 2972–2982, 2002; 10.1152/jn.00631.2001. We studied γ-aminobutyric acid (GABA)-mediated currents in short-term cultured dorsal unpaired median (DUM) neurons of cockroach Periplaneta americana using the whole cell patch-clamp technique in symmetrical chloride solutions. All DUM neurons voltage-clamped at −50 mV displayed inward currents (I\(_{\text{GABA}}\)) when 10\(^{-4}\) M of GABA was applied by pneumatic pressure-ejection pulses. The semi-logarithmic curve of I\(_{\text{GABA}}\) amplitude versus the ejection time yielded a Hill coefficient of 4.0. I\(_{\text{GABA}}\) was chloride (Cl\(^{-}\)) because the reversal potential given by the current-voltage (I-V) curve varied according to the reversal value predicted by the Nernst equation for Cl\(^{-}\). In addition, I\(_{\text{GABA}}\) was almost completely blocked by bath application of the chloride channel blockers picrotoxin (PTX) or 3,3-bis(trifluoromethyl)bicyclo-[2,2,1]heptane-2,2-diacarbonitrile (BIDN). The I-V curve for I\(_{\text{GABA}}\) displayed a unexpected biphasic aspect and was best fitted by two linear regressions giving two slope conductances of 35.6 ± 2.1 and 80.9 ± 4.1 nS for potentials ranging from 0 to −30 and −30 to −70 mV, respectively. At −50 mV, the current amplitude was decreased by cadmium chloride (CdCl\(_2\), 10\(^{-4}\) M) and calcium-free solution. The semi-logarithmic curve for CdCl\(_2\)-resistant I\(_{\text{GABA}}\) gave a Hill coefficient of 2.4. Hyperpolarizing voltage step from −50 to −80 mV was known to increase calcium influx through calcium-resting channels. According to this protocol, a significant increase of I\(_{\text{GABA}}\) amplitude was observed. However, this effect was never obtained when the same protocol was applied to cell body pretreated with CdCl\(_2\). The calmodulin blocker N-(6-aminohexyl)-5-chloro-1-naphthalene-sulfonamide or the calcium-calmodulin-dependent protein kinase blocker 1-[(O-bis(5-isoquinolinesulfonfonyl)-N-methyl-1-tyrosyl)-4-phenylpiperazine (KN-62) was added in the pipette solution, I\(_{\text{GABA}}\) amplitude was decreased. Pressure ejection application of the cis-4-aminocrotonic acid (CACA) on DUM neuron cell body held at −50 mV, evoked a Cl\(^{-}\)-inward current which was insensitive to CdCl\(_2\). The Hill plot yielded a Hill coefficient of 2.3, and the I-V curve was always linear in the negative potential range with a slope conductance of 32.4 ± 1.1 nS. These results, similar to those obtained with GABA in the presence of CdCl\(_2\) and KN-62, indicated that CACA activated one subtype of GABA receptor. Our study demonstrated that at least two distinct subtypes of Cl\(^{-}\)-dependent GABA receptors were expressed in DUM neurons, one of which is regulated by an intracellular Ca\(^{2+}\)-dependent mechanism via a calcium-dependent protein kinase. The consequences of the modulatory action of Ca\(^{2+}\) in GABA receptors function and their sensitivity to insecticide are discussed.

INTRODUCTION

γ-aminobutyric acid (GABA)-gated chloride channel receptors are largely widespread in the CNS of insects where their physiological role is to mediate fast inhibitory neurotransmission (Anthony et al. 1993; Sattelle 1990). Although insect ionotropic receptors have been shown to share some functional analogies with their vertebrate counterparts, many studies have demonstrated that insect GABA-operated Cl\(^{-}\) channels are pharmacologically and structurally distinct from vertebrate GABA\(_A\) receptors (Sattelle et al. 1991). In addition, the ionotropic GABA receptors described in insect CNS are of considerable interest because they form an important molecular targets for distinct chemicals classes of insecticidally active compounds such as picrotoxin (PTX), dieldrin, fipronil, and BIDN (Bloomquist 1996; Eldefrawi and Eldefrawi 1987; Sattelle 1990). In these regards, differences between vertebrate and insect GABA receptors have always been proved to be exploited for designing novel more selective insecticide molecules. During the last decade, molecular biology studies of insect ionotropic GABA receptors have contributed not only to further understand their structural and functional organization but also to support their heterogeneity. To date, three GABA receptors subunit genes have been cloned from Drosophila melanogaster including the resistance to dieldrin Rd1 gene (FFrench-Constant et al. 1993), the ligand-gated chloride channel homologue 3, LCC3 β-like gene (Henderson et al. 1993), and the glycine-like receptor GRD gene (Harvey et al. 1994). In addition, 12 GABA\(_A\)/glycine-like receptor subunit genes have been recently identified in the D. melanogaster genome (Rubin et al. 2000) confirming the existence of a multiplicity of GABA receptor subunits in insects.

Neuronal cell lines and oocyte expression system have been mainly used for establishing the pharmacological properties of insect recombinant RDL receptors (Buckingham et al. 1994a; Grolleau and Sattelle 2000; Hosie and Sattelle 1996; Millar et...
Insect neuronal preparations have also been developed that allowed studies on native GABA receptors (Aydar and Beadle 1999; Buckingham et al. 1994b; Dubreil et al. 1994; Hue 1991; Lees et al. 1987; Sattelle 1990; Shimahara et al. 1987; Watson and Salgado 2001; Zhang et al. 1994). For instance, in cockroach CNS, bicuculline-insensitive GABA receptors coupled to Cl\(^{-}\) channels have been detected in a ventral giant interneuron (GI) (Hue 1991), in an identified motor neuron Df (Buckingham et al. 1994b; David and Pitman 1996) and in dorsal unpaired median (DUM) neuron (Dubreil et al. 1994; Goodman and Spitzer 1980; Le Corronc and Hue 1999). In GI preparation, two GABA-gated Cl\(^{-}\) channel receptor subtypes have been further distinguished on the basis of their differences in Cl\(^{-}\) conductance and GABA and picrotomin sensitivity (Hue 1998). Although the presence of two GABA receptor-operated Cl\(^{-}\) channel subtypes have been hypothesized in cockroach DUM neurons (Le Corronc and Hue 1999), the detailed electrophysiological and pharmacological properties of these receptor subtypes remain to be investigated.

In addition, there is now substantial evidence that intracellular messengers and regulatory proteins can modulate vertebrate GABA-activated Cl\(^{-}\) currents (Moran and Dascal 1989). In this context, phosphorylation/dephosphorylation process is currently regarded as an important mechanism for modulating the function of GABA receptors (Moss et al. 1992; Raymond et al. 1993; Swope et al. 1999) and consequently for controlling synaptic plasticity (Smart 1997). Many GABA receptor subtypes contain consensus sites for phosphorylation (Shofield et al. 1987), and most intracellular regulatory mechanisms, described for vertebrate GABA\(_A\) or GABA\(_B\) receptors, involved Ca\(^{2+}\)-sensitive proteins such as protein kinase C, Ca\(^{2+}\)/calmodulin (CaM)-dependent protein kinase II (CaM K II), and/or calcineurin (Browning et al. 1990; Chen et al. 1990; Feigenspan and Bornmann 1994; Filippova et al. 1999; Huang and Dillon 1998; Krishek et al. 1994; Swope et al. 1999). Functional studies have demonstrated that phosphorylation might lead to depression or potentiation of the GABA response according to the subunit composition of receptors, their locations (synaptic or extrasynaptic), and the type of cells which expressed the receptors. In contrast to vertebrate, excepting a few studies that have been published elsewhere in abstract form (Alix et al. 2000).

**Methods**

**Preparation**

All experiments were performed on DUM neuron cell bodies isolated from the dorsal midline of the terminal abdominal ganglion (TAG) of the nerve cord of adult male cockroach *Periplaneta americana*. Insects are taken from our laboratory stock colonies maintained at 29°C with a photoperiod of 12 h light:12 h dark. Cockroaches were immobilized dorsal side up on a dissection dish. The dorsal cuticle, gut, and some dorsolongitudinal muscles were removed to allow access to the ventral nerve cord. The TAGs were carefully dissected and placed in normal cockroach saline containing (in mM) 200 NaCl, 3.1 KCl, 5 CaCl\(_2\), 4 MgCl\(_2\), 50 sucrose, and 10 HEPES; pH was adjusted to 7.4 with NaOH.

**Cell isolation**

Isolation of adult DUM neuron cell bodies was performed under sterile conditions using enzymatic digestion and mechanical dissociation of the median parts of the TAG as previously described (Grolleau and Lapied 1996; Lapied et al. 1989). Briefly, the dorsal median parts were incubated for 40 min at 29°C in cockroach saline containing collagenase (type IA, 1.5 mg/ml, Worthington Biochemical). The ganglia were then rinsed twice in normal saline and mechanically dissociated by repetitive gentle suction through fire-polished Pasteur pipettes. The DUM neurons, suspended in normal saline supplemented with fetal calf serum (5% by volume, Life Technologies, Cergy Pontoise, France), penicillin (50 IU/ml), and streptomycin (50 µg/ml), were allowed to settle on poly-D-lysine hydrobromide (MW, 70,000–150,000, Sigma Chemicals) coating the bottom of 35-mm tissue-culture petri dishes.

**Electrophysiological recordings**

Whole cell patch-clamp recording (Hamill et al. 1981) was carried out in DUM neuron cell bodies 24 h after dissociation. Only those having diameter of ~60 µm and a bright appearance under phase contrast microscope were selected. The GABA-induced currents were recorded using an Axopatch 200A amplifier (Axon Instruments, Foster City, CA) and filtered at 5 kHz (~3 dB, 4-pole low-pass Bessel filter). Patch pipettes were pulled from borosilicate glass capillary tubes (Clark Electromedical Instruments, Reading, UK) with a Narishige puller (PP-83, Tokyo, Japan) and had resistance ranging from 0.9 to 1.2 MΩ when filled with the internal solution (see composition in the following section). The liquid junction potential between bath and internal pipette solution was always corrected before the formation of a gigapshm seal (~3 GΩ). Signals were stored on-line on the hard disk of a NEC celeron 333 computer connected to a 125-kHz labmaster DMA acquisition system (TL-1–125 interface, Axon Instruments). The pClamp package (version 6.04, Axon Instruments) was used for data acquisition and analysis (sampling frequency, 2 kHz). When necessary, a SMP-300 programmable stimulator (Biologic, Echirolles, France) was used to apply hyperpolarizing pulses.

**Solutions and chemicals**

The cells were continuously superfused with a Cl\(^{-}\)-isotonic solution (in mM): 167 NaCl, 33 d-gluconic acid, 3.1 KCl, 4 MgCl\(_2\), 5 CaCl\(_2\), and 10 HEPES; pH was adjusted to 7.4 with NaOH. The saline was supplied by a gravity perfusion system at a constant rate (0.1 ml/min) through a plastic tubing positioned near the cell body (~100 µm). The pipette solution consisted of (in mM) 170 KCl, 15 NaCl, 1 MgCl\(_2\), 0.5 CaCl\(_2\), 3 ATP-Mg, 10 EGTA, 20 HEPES, and 10 phosphocreatine diTris; pH was adjusted to 7.4 with KOH. Antagonists [picrotoxin, 3,3-bis(tri fluoromethyl)bicyclo-[2,2,1]heptane-2,2-di carbonitrile (BIDN), cadmium chloride] were diluted in the bathing solution and modulators of the secondary effectors [N-(6-amino hexyl)-5-chloro-1-naphthalene-sulfonamide (W7) and 1-[N,O-bis(5-isquinolinesulfonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine (KN-62)] were added in the internal solution. Stock solution of BIDN, W7, and KN-62 were previously dissolved in dimethylsulfoxide (DMSO). The final concentration of DMSO never exceeded 0.1%. For extracellular
Ca^{2+}-free solution, MgCl_2 was substituted for CaCl_2 in equivalent amount. BIDN was obtained from DuPont Agrochemical. All other pharmacological agents and chemicals were obtained from Sigma Chemicals (L’Isle d’Abeau Chesnes, France). Experiments were carried out at room temperature (20°C).

Pneumatic pressure ejection application of agonist and data analysis

GABA (10^{-4} M) and CACA (10^{-3} M) were dissolved in the extracellular saline solution and were delivered by pressure ejection (15 lb./in.² gauge) with a pneumatic pressure ejection system (Miniframe PPS-2, Medical System, Greenvale, NY). Agonists were ejected through a glass micropipette (resistance, 2 MΩ when filled with agonist) placed at ~40 μm of the cell body. When droplets were ejected under oil and the diameter was measured with an ocular micrometer, a linear relationship was established between the volume delivered and the pulse duration parameters (McCamant et al. 1977). Consequently, we constructed an agonist dose-response relationship by increasing the ejection time at constant pressure. In these conditions, we assumed that the amount of agonist delivery by pressure was linearly related to ejection pressure (Di Angelotonio and Nistri 2001; Lapied et al. 1990; McCaman et al. 1977; Raymond et al. 2000). Pressure application from fine-tipped micropipette was preferentially used to apply agonist to minimize the risk of desensitization and to avoid large exposition of all other cells in the chamber. In no experiment did the pressure ejection of normal saline affect the current baseline. The steady-state recordings were made 2 min after setting of the whole cell recording configuration and repeated applications of GABA were made with an interval of 2 min between the end of one application and the beginning of the next. Under these conditions, amplitude of \( I_{\text{GABA}} \), normalized to the value of the response to 120-ms pulse duration, was then plotted against increasing pulse duration. The dose-response curves were analyzed using GraphPad Prism and were fitted to a sigmoid function with four-parameter logistic equation (sigmoid concentration response) with a variable slope. The equation used to fit the concentration-response relationship was

\[
I = I_{\text{max}}[1 + (T_{\text{off}}/T)^n]
\]

where \( I \) was the normalized GABA-activated current, \( I_{\text{max}} \) was the maximal normalized GABA current, \( T \) was the duration of the pulse, \( T_{\text{50}} \) was the pulse duration inducing 50% of the \( I_{\text{max}} \), and \( n \) the Hill slope factor. Dose-response curves were obtained from the mean of the normalized values calculated for each neuron. Data are expressed as means ± SE. Using a nonparametric Student’s \( t \)-test assessed statistical significance of data.

RESULTS

Transient inward current evoked by pressure ejection application of GABA

Whole cell recordings were performed in symmetrical chloride ion solutions ([Cl]_o = [Cl]_i = 188 mM). In all DUM neuron cell bodies tested, GABA induced a transient inward current at a holding membrane potential of ~50 mV. The amplitude of the inward current increased when the GABA dose was elevated by progressively raising the length of the pressure ejection pulse. Figure 1A illustrates typical examples of GABA-activated currents (\( I_{\text{GABA}} \)) evoked by pulse of various duration (from 30 to 300 ms). Normalized amplitudes of \( I_{\text{GABA}} \) were plotted against the logarithm of increasing pulse duration (Fig. 1B). A \( T_{\text{50}} \) value of 86.2 ms was revealed [-++, which corresponded to the best fit through the mean data points

\[
\text{slope} = 4.0
\]

\( r = 0.9995 \) according to the Hill equation (see METHODS). The Hill slope factor determined by a linear regression analysis of the Hill plot (Fig. 1B, inset) was 4.0 ± 0.2 (n = 9, \( r = 0.9995 \)). The amplitude of the inward current was maximum for pressure ejection duration >200 ms (Fig. 1B). In the following experiments, the pulse duration was adjusted to give a half-maximal response (e.g., 80 ms). In these conditions, the amplitude of \( I_{\text{GABA}} \) evoked by repeated puffs separated by 2 min remained stable over 30 min.

Voltage dependence and ionic selectivity of \( I_{\text{GABA}} \)

Figure 2Aa shows inward currents activated by a 80-ms pulse of GABA recorded at different steady-state membrane

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\]
potentials ranging from −70 to +30 mV in 10-mV increments. The relationship between amplitude of $I_{\text{GABA}}$ and membrane potential ($I$-$V$ curve) was constructed by averaging data from a total of 12 cells (Fig. 2Ab). The $I$-$V$ curve exhibited inward rectification at positive membrane potentials and gave a reversal potential of +1.8 mV. The curve shows that the currents were inwardly directed for potentials more negative than +1.8 mV and became outward above −30 mV. The reversal potential (+1.8 mV) was very close to the calculated $E_{\text{Cl}}$ (−0.1 mV) with symmetrical [Cl] inside and outside the cell (●) but shifted to −66 mV when [Cl] in the patch pipette was reduced to 13 mM (○). $Ba$: representative current evoked by 80-ms pulse of GABA (10−4 M) at −50 mV before, during, and after application of either 10−6 M 3,3-bis(trifluoromethyl)bicyclo[2,2,1]heptane-2,2-diacarbonitrile (BIDN; top) or 10−4 M picrotoxin (bottom). The current did not fully recover following the washout of each blocker.

**Effect of Ca2+ ions on $I_{\text{GABA}}$**

As mentioned in INTRODUCTION, we were interested by examining the role of Ca2+ in the regulation of $I_{\text{GABA}}$. Consequently, we next tested the effect of bath saline containing CdCl2 or Ca2+-free solution on DUM neuron cell body held at −50 mV. The amplitude of $I_{\text{GABA}}$ was decreased by 42.9 ± 4.1% ($n = 13$; $P < 0.01$) under CdCl2 (10−3 M). This effect occurred within 6 min and was reversible. Treatment of the cell with Ca2+-free solution also reduced the current by 26.0 ± 7.1% ($n = 3$; $P < 0.05$; Fig. 3A, a and b). Figure 3B shows the dose/response relationship constructed under 10−3 M CdCl2; the — corresponds to the best fit through the mean data points ($r = 0.9993$). The mean value of the Hill coefficient was estimated to be 2.4 ± 0.1 ($n = 6$), a value that was much smaller than one determined under control condition (see Fig. 1B). In the literature, it is well documented that two molecules of GABA are necessary for activation of the native receptor channel (MacDonald and Olsen 1994). In our case, it was unclear if the high Hill slope factor value (4.0) calculated under control conditions resulted of the simultaneous binding of more than two molecules of GABA or if two different GABA receptor subtypes could be involved in the GABA-induced current. Based on results obtained under CdCl2 treatment, it
was suggested that two receptor subtypes were expressed in DUM neurons. However, only one of them could be still activated in the presence of CdCl₂. To confirm this hypothesis, we compared the I-V curve between -70 and 0 mV in control and under CdCl₂ (10⁻³ M). In the last case, it was clear that the voltage dependence of \( I_{\text{GABA}} \) did not display the biphasic aspect (Fig. 3C) as described in the preceding text. Although two linear regressions were necessary to fit mean data points in control (Table 1), only one linear regression was used under CdCl₂ treatment between 0 and -70 mV (Fig. 3C and Table 1). Furthermore, the parameters of the linear regression used under CdCl₂ were very similar to that of the control between 0 and 30 mV (see Table 1 for more details).
These authors demonstrated that increases of intracellular calcium, in the GABA receptor, was hyperpolarized from resting channels were observed in DUM neurons, when the holding potential was −30 mV. In conclusion, comparisons of both Hill coefficient values together with the I/V curves obtained in control and under CdCl₂ argued for the activation of one of the two different receptor subtypes.

The restricted negative potential range in which CdCl₂ was active suggested a possible implication of CdCl₂-sensitive calcium resting channels, known in DUM neurons to be mainly activated in the hyperpolarizing potential range between −50 and −110 mV (Heine and Wicher 1998; Wicher et al. 1994). These authors demonstrated that increases of intracellular calcium concentration following opening of Ca²⁺ resting channels were observed in DUM neurons, when the holding potential was hyperpolarized from −50 to −70 or −90 mV (Heine and Wicher 1998). Accordingly, it was decided to stimulate Ca²⁺ entry through these Ca²⁺ resting channels by stepping the membrane from −50 to −80 mV for 1 min before ejecting GABA. I_GABA were recorded within 3 s after the termination of the hyperpolarizing steps. In all cell tested, I_GABA were always significantly potentiated by 20.8 ± 4.9% (n = 6; P < 0.01; Fig. 3D) with a recovery period of −4 min. By contrast, when the same hyperpolarizing voltage step was applied in the presence of CdCl₂ (10⁻³ M), amplitude of I_GABA recorded at −50 mV did not increase (2.2 ± 1.4% of reduction, n = 3, P > 0.1, Fig. 3D). The histogram illustrated in Fig. 3E summarizes these results. It clearly shows that one of the components of I_GABA at −50 mV was dependent on Ca²⁺ entry through Ca²⁺ resting channels.

**Implication of Ca-dependent proteins for maintaining I_GABA**

The Ca²⁺ sensitivity of the GABA response might be explained by either a direct interaction of Ca²⁺ with the GABA receptors or by an activation of Ca-dependent enzymatic processes that co-activate the GABA receptors. Because direct effect of Ca²⁺ ions on GABA receptors have been reported in vertebrate to be mainly involved in the suppression of GABA-activated current (e.g., Inoue et al. 1986; Martina at al. 1994) rather than a potentiation of the response (our study), we performed additional experiments using an internal pipette solution containing Ca²⁺-dependent messenger inhibitors. Calmodulin is a ubiquitous intracellular protein that regulates the activity of various enzymes in a Ca²⁺-dependent manner. To explore the putative participation of calmodulin in DUM neuron, I_GABA was recorded at a holding potential of −50 mV using a pipette solution containing 10⁻³ M of the calmodulin inhibitor W7. In this condition, the recordings were performed over a time period of 30 min after establishing the whole cell configuration to allow compounds sufficient time to diffuse into the cell. I_GABA was then normalized to the value measured 4 min after the initial GABA application. In five cells tested, the main effect obtained with W7 after 20 min consisted of a reduction of I_GABA (17.7 ± 2.7%, P < 0.05). The plot of the mean values of I_GABA versus time was illustrated in Fig. 4A. However, in three cells, I_GABA was increased by 56.0 ± 7.5% and in other three cells, there was no variation (3.0 ± 1.0%).

The current amplitude over time in the absence of the drug (●, Fig. 4A) did not change and had a value of 97.3 ± 5.1% of its initial value after 20 min (n = 5). Because heterogeneous effects were obtained with W7, we tested the effect of the specific inhibitor of the Ca²⁺/calmodulin-dependent protein kinase II (CaM kinase II), the KN-62. At a holding potential of −50 mV, KN-62 (5.10⁻⁶ M) reduced I_GABA by 38.3 ± 8.4% (n = 9, P < 0.05) within 20 min in all cells tested (Fig. 4B).

The voltage dependence of I_GABA was studied in the presence of 5.10⁻⁶ M KN-62. As shown in Fig. 4C, the discontinuity of the I/V curve disappeared in the presence of KN-62, and data were fitted by a single linear regression between 0 and −70 mV, yielding a slope factor close to those previously obtained under CdCl₂ and in control condition (Table 1). In addition, when CdCl₂ was tested on the remaining current under KN-62 (Fig. 4D), no additional reduction of I_GABA was observed (99.5 ± 5.4%, n = 4, P > 0.1). The results indicated that the CaM kinase II-like protein was implicated in the regulation of the CdCl₂-sensitive component of I_GABA.

**Activation of only one component by CACA**

To substantiate the hypothesis for the co-existence of two distinct subtypes of GABA Cl⁻-gated receptor in DUM neurons, the cis-4-aminoacetonitrile acid (CACA), the well known agonist of vertebrate GABA_A receptor (Johnston 1996) and insect GABA receptor such as RDL receptor (Millar et al. 1994) or native giant interneuron GABA receptor (Hue 1998), was tested. Pressure application of 10⁻³ M CACA, for various ejection duration pulses, onto the isolated cell body (holding potential −50 mV), elicited a transient inward current (Fig. 5A). The amplitudes of the peak current (I_CACA) were normalized to the value of the response to 300-ms ejection pulse for each cell. The mean data points (n = 4) were then plotted against the logarithm of increasing pressure ejection duration (Fig. 5B). The solid line corresponded to the best fit (correlation coefficient r = 0.9996) according to the Hill equation. The mean value of the Hill coefficient, determined by a linear regression analysis of the Hill plot (Fig. 5B, inset) was 2.3 ± 0.1 and corresponded to a value close to that already calculated for I_GABA under CdCl₂ treatment. In addition, as illustrated in Fig. 5C, 10⁻³ M CdCl₂ did not significantly reduce the response evoked by 200-ms pulse of CACA (5.1 ± 1.9%, n = 3, P > 0.05). The I/V plot of the current evoked by CACA, constructed from five cells, confirmed that the CACA-induced

### Table 1. **Slope conductance**

<table>
<thead>
<tr>
<th>No. of linear regressions</th>
<th>Control</th>
<th>10⁻³ M CdCl₂</th>
<th>5.10⁻⁶ M KN-62</th>
<th>10⁻³ M CACA</th>
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<tr>
<td>Potential range, mV</td>
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<td></td>
<td></td>
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<tr>
<td>0 to −30</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1</td>
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<tr>
<td>−30 to −70</td>
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<tr>
<td>No. of cells</td>
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<td>13</td>
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<tr>
<td>Slope, nS</td>
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<tr>
<td>35.6 ± 2.1</td>
<td>0.9965</td>
<td>0.9962</td>
<td>0.9962</td>
<td>0.9985</td>
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<tr>
<td>80.9 ± 4.1</td>
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<tr>
<td>r</td>
<td>0.9962</td>
<td>0.9962</td>
<td>0.9985</td>
<td>0.9962</td>
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</table>

Slope conductance was calculated from the linear regressions of the I-V relationships obtained for GABA [in control condition and in the presence of CdCl₂ or 1-[N,O-bis(5-isouquinolinesulfonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine (KN-62)] and for cis-4-aminoacetonitrile acid (CACA). Values are means ± SE.

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current was chloride (Fig. 5D). The estimated reversal potential was \( E_{\text{Cl}} \) = 3.5 mV, a value very close to \( E_{\text{Cl}} \) in our experimental conditions. However, the I-V curve did not display the biphasic aspect between \(-10 \) and \(-70 \) mV (see Table 1) but showed a voltage dependence that was similar to those observed with CdCl\(_2\) and KN-62. These results support the idea that two different receptor subtypes contributed to the global inward current evoked by GABA at \(-50 \) mV. Either GABA or CACA might activate one receptor subtype, shown to be not regulated by a CaM kinase II-like protein.

**DISCUSSION**

The present study provides evidences for the existence in DUM neuron cell body of more than one ionotropic GABA receptor subtype including a Cl\(^{-}\)-dependent GABA receptor that is regulated by an intracellular Ca\(^{2+}\)-dependent mechanism.

**Evidence for the co-existence of two distinct Cl\(^{-}\)-dependent GABA receptor subtypes**

Although the voltage dependence of the whole cell response evoked by GABA usually results in a uniphasic I-V relationship in vertebrates (e.g., Filippova et al. 1999; Kapur et al. 1999; Tietz et al. 1999) as well as in insect (e.g., Wafford and Sattelle 1986; Zhang et al. 1994), our results show a more complex I-V curve because a discontinuity was observed for negative potentials. The reversal potential is very close to the equilibrium potential for Cl\(^{-}\) ions, indicating that the current is chloride. This conclusion, reinforced by the complete blocking effect of both PTX and BIDN, indicated that \( I_{\text{GABA}} \) is mediated by an ionotropic Cl\(^{-}\)-dependent GABA receptor and not a GABA\(_B\) receptor. Although an unequal chloride activity across the membrane would be responsible for the rectification property, it was suggested that more than one conductance probably underlie the discontinuity of the I-V relationship. Three sets of experiment have been performed to determine the number of receptor subtypes involved. First, the dose-response curve that have been established at \(-50 \) mV by varying the pressure ejection time (see METHODS) yielded a high Hill coefficient (i.e., 4). Second, analysis of the pharmacological profile indicated that the inward current recorded at a membrane potential more negative than \(-30 \) mV could be further dissociated into two components including a Ca\(^{2+}\)-sensitive Cl\(^{-}\) current and a Ca\(^{2+}\)-insensitive Cl\(^{-}\) current. Finally, we found that only the Cd\(^{2+}\)-insensitive component of \( I_{\text{GABA}} \) is activated by the GABA\(_C\) agonist, CACA. However, CACA was \( \geq 10 \)-fold weaker as a agonist to elicit the Ca\(^{2+}\)-insensitive Cl\(^{-}\) current in DUM neurons and consequently, it was difficult to assess a relative contribution of both receptors. Although it was never demonstrated to our knowledge that Hill coefficients should be additive, we conclude that at least two GABA receptor types are expressed in DUM neuron cell body. In this context, a bimodal dose-response curve for the combined current would be expected. Instead, the Hill analysis suggested that the two
receptors have identical dissociation constant and showed a degree of positive cooperativity comparable to that reported for instance for DUM neuron cholinergic receptors (i.e., $n_{\text{Hill}}$ = 4.28) (Lapied et al. 1990). Among others possibilities, we can speculate that the high value of Hill coefficient may reflect the interaction of receptor molecules. In fact, there is growing evidence that a positive cooperativity exists between a few nearby receptors (Bornhorst and Falke 2000). Such cooperativity between receptors was proposed as an alternative of signal transduction and particularly in amplifying signal transduction events (Bornhorst and Falke 2000; Chen et al. 2000; Liu et al. 2000; Yonekura et al. 1991).

Based on the literature, the characterization of two different ionotropic GABA receptor subtypes within individual cell body seems to be unique in an insect neuronal preparation. To date, two kinds of picrotoxin-sensitive GABA receptor were differentiated according to their location on DUM neurons (Dubreil et al. 1994). It was shown that an extrasynaptic GABA receptor was located onto the soma of DUM neuron, and another synaptic receptor was also revealed on the neuritic arborization of these cells. In vertebrates, there are only few examples reporting that two Cl current mediated by different GABA receptors could be recorded in the same cell (e.g., Han and Yang 1999; Tietz et al. 1999). For instance, Tietz et al. (1999) suggested that the biphasic response to GABA from rat CA1 pyramidal cells reflected the presence of molecular heterogeneous GABA receptors. In our case and because the CdCl$_2$-resistant Cl current activated by GABA was also activated by CACA, it is tempting to assume that one of the two receptors identified might correspond to a GABAC-like receptor. The presence of such receptor subtype has previously been suggested in cockroach ventral giant interneuron (Hue 1999). Another possibility, which cannot be ruled out, is the putative existence of an RDL-like receptor on DUM neurons. Subunit composition of GABA receptors in cockroach CNS remains unknown. However, several studies have suggested that RDL-like receptor might exist in cockroach. First, expression of Rdl subunit in Xenopus oocytes or S2 cells give functional GABA-gated Cl channels exhibiting a pharmacological profile that shares many of the properties of native GABA receptors on cockroach giant interneuron (Buckingham et al. 1994b) or motor neuron Df (Sattelle 1990). In addition, immunocytochemical staining with polyclonal antibody raised against the predicted C-terminal sequence of the cloned RDL receptor has been found largely distributed in the cockroach head ganglia (Sattelle et al. 2000). It should be noted that the homo-oligo-

![Graph showing the relationship between normalized $I_{CACA}$ and ejection time with a slope of 2.3.]

![Graph showing the effect of CdCl$_2$ on CACA-induced currents.]

![Graph showing the voltage dependence of CACA-induced currents.]

![Graph showing the dose-response curve for CACA-induced currents.]

**Fig. 5.** cis-4-Aminocrotonic acid (CACA)-induced current ($I_{CACA}$) in isolated DUM neurons in Cl$^-$-isotonic conditions. A: typical examples of current traces recorded in response of increasing CACA doses at a holding membrane potential of $-50$ mV. CACA ($10^{-3}$ M) was delivered from the pipette at various pressure-pulse durations indicated next to each trace. Points correspond to the amplitude of $I_{CACA}$ measured on the same cell body for the different pressure-pulse durations and were normalized to the value obtained in response to the 300-ms pulse of CACA. —, the best fit of the mean data ($n = 4$) using a 4-parameter logistic equation. Inset: the corresponding Hill plot of data. —, the best fit to a linear regression with a slope of 2.3. B: CACA-induced currents recorded in the absence or presence of CdCl$_2$ ($10^{-3}$ M). DUM neuron was clamped at $-50$ mV and CACA ($10^{-3}$ M) was applied for a duration of 200 ms. D: voltage dependence of the currents induced by 200-ms pulses of CACA. Linear regression through the mean data points gave a reversal potential of $-2$ mV and yielded a slope which is close to that obtained for GABA in control between 0 and $-30$ mV (see Table 1). Inset: superimposed whole cell currents evoked by locally applied CACA at various membrane holding potentials increasing in 20-mV increments from $-70$ to $+10$ mV.
Ca\(^{2+}\)/calmodulin-dependent protein kinase regulated one component of \(I_{\text{GABA}}\)

Overall, our data demonstrate that Ca\(^{2+}\) is necessary to maintain one component of \(I_{\text{GABA}}\). We have found that CdCl\(_2\) or Ca\(^{2+}\)-free solution inhibits the GABA response. Conversely, \(I_{\text{GABA}}\) is increased when the intracellular Ca\(^{2+}\) concentration is raised artificially. This has been confirmed by using hyperpolarizing voltage step known to increase Ca\(^{2+}\) influx through Ca\(^{2+}\) resting channels (Heine and Wicher 1998). Amplitude of \(I_{\text{GABA}}\) was dependent either of increase or decrease of intracellular Ca\(^{2+}\) concentration. These observations together with the reversibility of the blocking action of Cd\(^{2+}\) on \(I_{\text{GABA}}\) suggest that Ca\(^{2+}\) did not likely involved through proteolysis of GABA receptor by Ca\(^{2+}\)-dependent proteases. In vertebrates, many studies have described an effect of variation in intracellular Ca\(^{2+}\) concentration on the GABA\(_{\alpha}\) receptor-gated Cl\(^{-}\) current (Akaike 1990; Inoue et al. 1986; Llano et al. 1991; Martina et al. 1994; Mouginot et al. 1991). In these cases, it was suggested that Ca\(^{2+}\) changed the apparent affinity of the receptor or acted through a diffusible Ca\(^{2+}\)-dependent messenger. In our study, because the effect induced by Cd\(^{2+}\) was mimicked by W7 or KN-62, which are specific inhibitors of calmodulin and CaM kinase II, respectively, we concluded that it occurred through a CaM kinase II-like protein.

Phosphorylation mechanisms were well documented in vertebrates as a major intracellular pathway that regulates neurotransmitter receptor function (Raymond et al. 1993; Swope et al. 1999). Conserved serine and threonine residues on the GABA\(_{\alpha}\) receptor \(\beta\) and \(\gamma\) subunits respectively have been identified as a site of protein phosphorylation by PK\(_{A}\), PK\(_{C}\), and CaM kinase II showing the importance of these subunits in GABA receptor activity (Krisheck et al. 1994; MacDonald and Olsen 1994; McDonald and Moss 1997; Raymond et al. 1993; Swope et al. 1999). The effects of PK\(_{C}\) or PK\(_{A}\) on GABA\(_{\alpha}\) receptors have been shown to involve a positive as well as a negative regulation depending on the specific preparation and probably on the existence of receptors composed of different subunits. By contrast, only few reports demonstrated that CaM kinase II protein is important for receptor activation. In rat acutely isolated spinal neurons, it has been shown that injection of CaM kinase II enhanced GABA-induced Cl\(^{-}\) current (Wang et al. 1995). This effect was associated with a reduction of the desensitization of the GABA response. In forebrain synaptosomal membranes, Ca\(^{2+}\) and CaM kinase II were implicated in the enhancement of the binding of agonist (Churn and Delorenzo 1998). In mouse cortical neurons, regulation of GABA\(_{\alpha}\) receptor function also involved a CaM kinase II (Aguayo et al. 1998). These authors demonstrated that both calmodulin and CaM kinase II inhibitors blocked the effect of increasing intracellular Ca\(^{2+}\) on responses to GABA.

Functional significance and interest in pest control strategy

We have demonstrated in this study that the GABA response was sensitive to changes in intracellular Ca\(^{2+}\) concentration. From these results emerges an interesting question concerning the relationship between the rise in intracellular Ca\(^{2+}\) concentration and the function of the receptor in the control of the membrane potential. Of particular interest is the consequence on the firing pattern because DUM neurons are well characterized by an endogenous pacemaker activity, which is closely related to their neurosecretory function (Grolleau and Lapied 2000). Among the different ionic currents underlying this pacemaker activity, several classes of voltage-dependent ionic currents have been identified that are regulated by change in intracellular Ca\(^{2+}\) concentration (Grolleau and Lapied 2000). Here it was demonstrated that an increase of intracellular Ca\(^{2+}\) concentration enhanced a GABA-gated Cl\(^{-}\) current through a CaM kinase II protein. Because the action of GABA is known to be inhibitory on the soma of DUM neuron (Dubreil et al. 1994; Goodman et Spitzer 1980; Washio 1994), such current would probably influence the resting membrane potential after various stimuli that elevate intracellular Ca\(^{2+}\) concentration, in a more hyperpolarizing direction compared with a Ca\(^{2+}\)-independent GABA receptor current. In this condition, the participation of the CaM kinase II-like-regulated GABA receptor will help in potentiating the inhibitory action of neurotransmitter on DUM neuron.

Because GABA receptors are one of major targets in insect for chloride channel-blocking insecticidal active molecules such as cyclohexane, cyclodiene, or fipronil (Bloomquist 1996; Hainzl et al. 1998), the evidence of phosphorylation mechanism for maintaining insect GABA receptor activation is of considerable importance for anticipating the action of insecticides but also for designing new molecules with insecticidal properties. The structure-activity relationship of GABA receptor binding insecticide molecules may be strongly dependent on the occurrence of phosphorylation system, and for this reason, significant difference in toxicity may be expected. For instance, it has been suggested from electrophysiological studies on drosophila S2 cell expressing RDL receptor (Grolleau and Sattelle 2000) or on rat DRG neuron (Ikeda et al. 2001) that receptor activation facilitates fipronil binding to the receptor. In this condition, involvement of CaM kinase II-like protein in keeping ionotropic GABA receptor fully operative should undergo change of insecticide effect in efficacy.

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